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Mitochondrial Calcium Signaling Driven by the IP_3 Receptor

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Many agonists bring about their effects on cellular functions through a rise in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) mediated by the second messenger inositol 1,4,5-trisphosphate (IP_3). Imaging studies of single cells have demonstrated that $[Ca^{2+}]_c$ signals display cell specific spatiotemporal organization that is established by coordinated activation of IP_3 receptor Ca^{2+} channels. Evidence emerges that cytosolic calcium signals elicited by activation of the IP_3 receptors are efficiently transmitted to the mitochondria. An important function of mitochondrial calcium signals is to activate the Ca^{2+}-sensitive mitochondrial dehydrogenases, and thereby to meet demands for increased energy in stimulated cells. Activation of the permeability transition pore (PTP) by mitochondrial calcium signals may also be involved in the control of cell death. Furthermore, mitochondrial Ca^{2+} transport appears to modulate the spatiotemporal organization of $[Ca^{2+}]_c$ responses evoked by IP_3 and so mitochondria may be important in cytosolic calcium signaling as well. This paper summarizes recent research to elucidate the mechanisms and significance of IP_3-dependent mitochondrial calcium signaling.

KEY WORDS: Mitochondria; endoplasmic reticulum; Ca^{2+}; IP_3; local signaling; energy metabolism; apoptosis; necrosis.

INTRODUCTION

It has been well established that mitochondria have a substantial capacity to accumulate Ca^{2+} and several Ca^{2+} transport mechanisms are involved in the control of mitochondrial Ca^{2+} homeostasis, but because of the relatively low affinity of mitochondrial Ca^{2+} uptake sites for Ca^{2+}, the physiological significance of mitochondrial Ca^{2+} transport has been the subject of controversy. Recently, experiments on cells expressing the Ca^{2+} sensitive photoprotein, aequorin in the mitochondria (Rizzuto *et al.*, 1993, 1994; Rutter *et al.*, 1996) and on single cells containing fluorescent Ca^{2+} probes within the mitochondria (Hajnoczky *et al.*, 1995; Donnadieu and Bourguignon, 1996; Csordas *et al.*, 1999) demonstrated that IP_3 produces increases in mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) that exceed changes of $[Ca^{2+}]_c$. Since elevations of the global $[Ca^{2+}]_c$ were too low to account for the IP_3-dependent $[Ca^{2+}]_m$ signals, it became a fundamental issue to explore the mechanisms underlying a privileged or local Ca^{2+} transfer between IP_3 receptors and mitochondrial Ca^{2+} uptake sites.

One important function of $[Ca^{2+}]_m$ elevations is in the control of mitochondrial oxidative metabolism via stimulation of dehydrogenase activity (Denton and McCormack, 1980; Hansford, 1980; McCormack *et al.*, 1990). Evidence in support of the activation of dehydrogenases by IP_3-dependent calcium signals derived from fluorescence imaging of the redox state of pyridine and flavin nucleotide cofactors simultaneously with $[Ca^{2+}]$ (Pralong *et al.*, 1994; Hajnoczky *et al.*, 1995). These studies also demonstrated that the complex spatiotemporal arrangements of $[Ca^{2+}]_c$ signals are transmitted to the mitochondria and are critical for shaping the $[Ca^{2+}]_m$ and metabolic responses. Although the mechanisms underlying integration of

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$[Ca^{2+}]_c$ signals into metabolic responses by the mitochondria have not, as yet, been completely understood, new areas of research have also been initiated since implications of mitochondrial calcium signaling in the control of cell death and in cytosolic calcium signaling also emerge.

A number of comprehensive reviews have been published recently on cytosolic calcium signaling (Berridge, 1993; 1997; Clapham, 1995; Thomas *et al.*, 1996), on mitochondrial Ca^{2+} transport (Gunter *et al.*, 1994; Pozzan *et al.*, 1994; Babcock and Hille, 1998; Jouaville *et al.*, 1998), on mitochondrial metabolism (McCormack *et al.*, 1990), and on the role of mitochondria in control of cell death (Bernardi, 1996; Reed, 1997; Green and Kroemer, 1998; Green and Reed, 1998; Ichas and Mazat, 1998; Kroemer *et al.*, 1998; Lemasters *et al.*, 1998; Skulachev, 1998). The present review is focused on the signaling mechanisms that are involved in control of mitochondrial functions by calcium signals originated from the IP_3 receptors. In particular, this work is concerned with the mechanism of Ca^{2+} transfer from IP_3 receptors to mitochondrial Ca^{2+} uptake sites and with encoding the specificity of mitochondrial responses. Before describing possible mechanisms that link together cytosolic and mitochondrial calcium signaling, a brief overview is given on the new tools, which allowed us to study mitochondrial signals at subcellular level.

VISUALIZATION OF MITOCHONDRIAL FUNCTION AT SUBCELLULAR RESOLUTION

During the past decade, development of the fluorescence imaging technology and the introduction of new fluorescent probes yielded critical progress in visualization of mitochondrial functions in single living cells. Figure 1 shows mitochondrial patterns in HepG2 cells visualized with green fluorescent protein (panel A), MitoTracker (panel B), fluorescein diacetate (panel C), tetramethylrhodamine ethyl ester (TMRE, panel D), and compartmentalized rhod2 (panel E). Green fluorescent protein targeted to the mitochondria (mitoGFP) has been used to visualize the subcellular distribution of mitochondria and the dynamics of mitochondrial movements (Rizzuto *et al.*, 1995; 1998, Fig. 1A). Fluorescence imaging of mitoGFP or other mitochondrion specific markers (*e.g.*, MitoTrackers) shows globular and tubular structures that may be arranged into a network with luminal continuity (Rizzuto *et al.*, 1998). The mitochondrial pattern is characteristic for each cell type and may be affected by a number of additional factors [*e.g.*, age (Ricken *et al.*, 1998)]. Interestingly, imaging of mitochondrial probes in living cells demonstrated rapid and continuous movements of the mitochondria (Rizzuto *et al.*, 1998). The mechanism underlying mitochondrial movements and the functional significance of the motion have not been elucidated. The GFP approach also allowed *in vivo* demonstration of close associations between endoplasmic reticulum (ER) and mitochondrial membranes (Rizzuto *et al.*, 1998) that may provide surface for local Ca^{2+} signal transmission from ER Ca^{2+} release sites to mitochondrial Ca^{2+} uptake sites.

The alkalinic pH in the mitochondrial matrix space (pH_m) and the highly negative mitochondrial membrane potential ($\Delta\Psi_m$) allow selective accumulation of dyes with net positive charge in the mitochondria or yield a characteristic fluorescence signal of the mitochondrial fraction of other dyes. These tools also facilitated visualization of mitochondrial distribution at subcellular resolution and allowed measurements of pH_m and $\Delta\Psi_m$ responses in cells exposed to various stimuli (Duchen *et al.*, 1993; Loew *et al.*, 1993; 1994; Di Lisa *et al.*, 1995; Kneen *et al.*, 1998; Llopis *et al.*, 1998; Robb-Gaspers *et al.*, 1998a,b). Figure 1C and D show mitochondrial distribution in HepG2 cells, visualized with a pH sensitive probe, fluorescein diacetate, and with a membrane potential sensitive probe, TMRE; also show an uncoupler-induced dissipation of the fluorescent signal deriving from the mitochondria (difference images shown in red).

Measurements of mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) in suspension of isolated mitochondria were accomplished first with Ca^{2+}-sensitive fluorescent dyes (quin2, fura2) introduced into mitochondria in the acetoxymethylester (AM) form (Lukacs and Kapus, 1987). After hydrolysis, the dye became trapped in the mitochondrial matrix. Studies on $[Ca^{2+}]_m$ in intact cells loaded with AM forms usually required many controls, since the dyes were trapped in more than one subcellular compartment, depending on the place of hydrolysis (cytosol, ER, mitochondria). Contamination of the mitochondrial signal with signals deriving from other organelles was avoided with targeted expression of aequorin in the mitochondria. Using this approach, Rizzuto, Pozzan, and co-workers demonstrated that mitochondria undergo a large $[Ca^{2+}]_m$ increase in response to stimulation with IP_3-linked stimuli in a wide variety of cells (Rizzuto *et al.*, 1993, 1994). Because of low signal levels, it has been difficult to

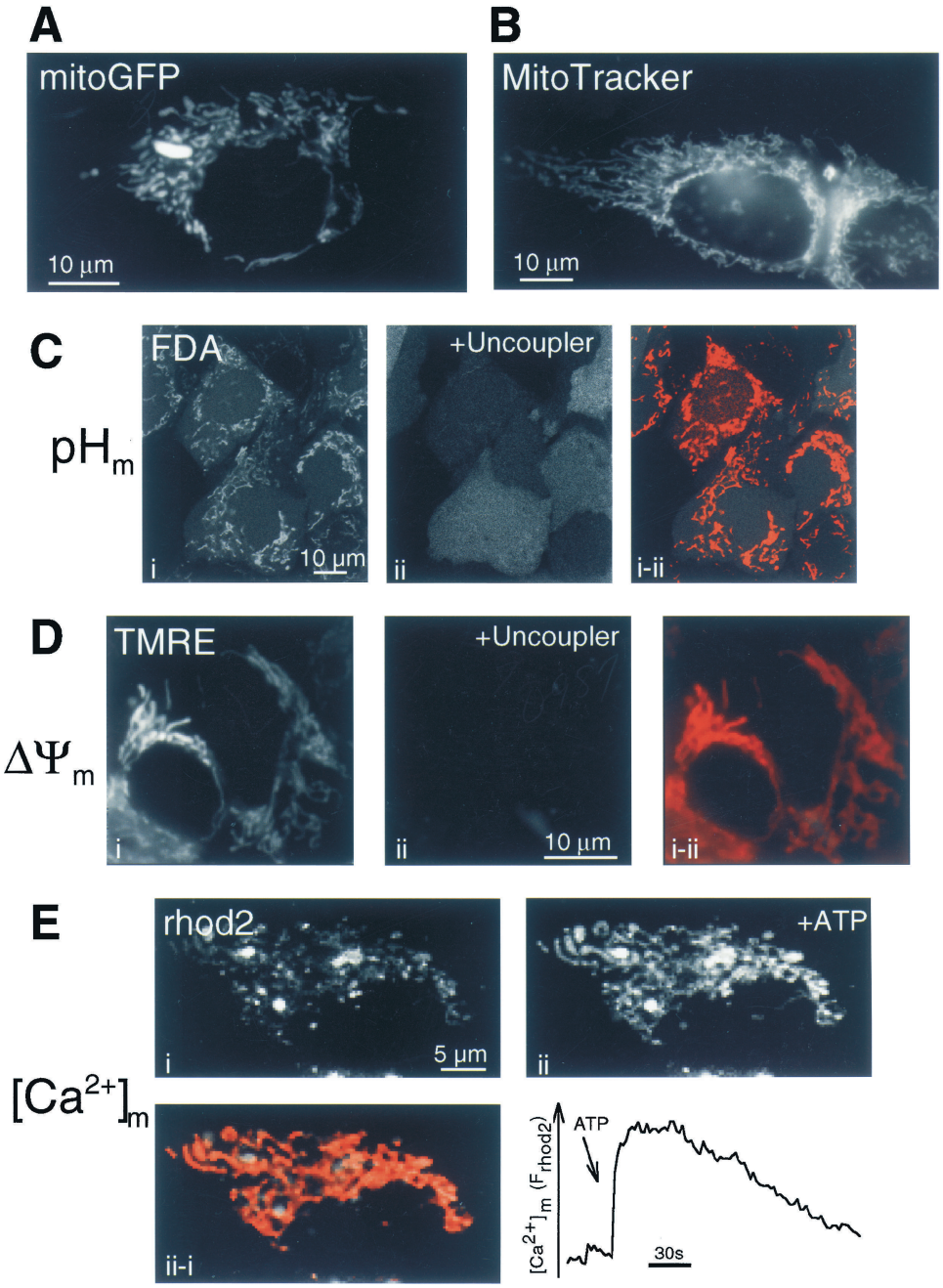

Fig. 1. Localization of mitochondria and measurement of $[Ca^{21}]_m$ in HepG2 cells. Fluorescence images of HepG2 cells transfected with mitoGFP (panel A), labeled with MitoTracker Green (panel B), with fluorescein diacetate (panel C), with TMRE (panel D), or loaded with rhod2/AM (panel E). Images (i) and (ii) in panels (C) and (D) show the fluorescence prior to and 7 min after addition of 5 mM uncoupler FCCP and 12.5 mg/ml oligomycin (1 uncoupler), respectively. The red overlays in images (iii) show the fluorescence change evoked by uncoupler, calculated by subtraction of (ii) from (i) images. In panel E, image (i) and (ii) show the fluorescence prior to and 30 s after addition of a supramaximal dose of ATP (200 mM), respectively. Image (iii) shows distribution of the $[Ca^{21}]_m$ response (red overlay), calculated by differentiation of (i) and (ii) images. Fluorescence imaging was carried out using a CCD camera system (panels A, B, and D) or a confocal microscope (panels C and E) as described previously (Hajnóczky *et al.*, 1995; Csordás *et al.*, 1999).

achieve single cell or subcellular resolution with aequorin expressed in the mitochondria and consumption of aequorin caused problems as well. Notably, selective labeling of the mitochondrial Ca^{2+} store with fluorescent Ca^{2+} probes has also been successful. Rhod2, a bright and stable Ca^{2+} probe has net positive charge in the AM form, which facilitates its sequestration into mitochondria due to potential-driven uptake (Tsien and Bacskai, 1995). Using rhod2 compartmentalized into the mitochondria, we achieved resolution of $[Ca^{2+}]_m$ at the single cell level and demonstrated that the pulsatile release of Ca^{2+} underlying $[Ca^{2+}]_c$ oscillations driven by the IP_3 receptor is delivered efficiently into the mitochondrial matrix, giving rise to coupled oscillations of $[Ca^{2+}]_m$ (Hajnoczky *et al.*, 1995). Figure 1E shows an example of imaging mitochondrial Ca^{2+} responses with compartmentalized rhod2 in cells stimulated by IP_3-linked agonists. Unfortunately, rhod2 is not a ratiometric dye and so calibration of the fluorescence of rhod2 in terms of $[Ca^{2+}]$ is difficult. Interestingly, some other fluorescent Ca^{2+} probes also show selective mitochondrial compartmentalization in certain cell types (*e.g.*, fura2FF in RBL-2H3 mast cells) that has allowed ratiometric measurements of $[Ca^{2+}]_m$ at high spatial and temporal resolution (Csordas *et al.*, 1999). Furthermore, $[Ca^{2+}]_m$ has also been measured at subcellular resolution with dyes that compartmentalized in more than one organelles using an organelle-specific probe simultaneously to localize the areas rich in mitochondria (Donnadieu and Bourguignon, 1996; Golovina and Blaustein, 1997).

In order to determine the links between calcium signaling and mitochondrial metabolism, it has been important to monitor fluorometrically the redox state of the pyridine (NAD(P)/NAD(P)H) and flavin nucleotides ($FAD/FADH_2$) (Kunz and Bohme, 1969; Scholz *et al.*, 1969; Balaban *et al.*, 1981). In particular, measurements of the activity of the Ca^{2+}-sensitive mitochondrial dehydrogenases (CSMDH), through fluorescence changes of the pyridine and flavin nucleotide cofactors, have been established at the level of single cells (Duchen, 1992; Pralong *et al.*, 1992, 1994; Hajnoczky *et al.*, 1995). Simultaneous measurements of $[Ca^{2+}]_c$ and NAD(P)H demonstrated that $[Ca^{2+}]_c$ oscillations are an effective signal for long-term activation of CSMDHs, whereas nonoscillatory $[Ca^{2+}]_c$ increases failed to sustain the activated state, although $[Ca^{2+}]_c$ remained at the same level achieved during the peak of the $[Ca^{2+}]_c$ oscillations (Hajnoczky *et al.*, 1995; Robb-Gaspers *et al.*, 1998a).

Taken together, these approaches to image mitochondria and mitochondrial functions at subcellular resolution provided important tools to explore the relation between cytosolic and mitochondrial calcium signaling. In addition to the complex spatiotemporal patterns displayed by IP_3-dependent cytosolic calcium signals, a number of properties of the mitochondria (*e.g.*, heterogeneous and dynamic distribution, requirement of high $[Ca^{2+}]$ to support Ca^{2+} uptake) became apparent to be involved in the subcellular organization of mitochondrial calcium signals. Improving resolution of the approaches described above and decreasing harmful effects associated with the imaging techniques (*e.g.*, light-induced generation of free radicals; Huser *et al.*, 1998) will be important to dissect the mechanism underlying local calcium signaling between ER and mitochondria.

SPATIOTEMPORAL ORGANIZATION OF CYTOSOLIC $[Ca^{2+}]$ RESPONSES

One of the most versatile and universal mechanisms of cellular signaling is through changes in the $[Ca^{2+}]_c$ (Berridge, 1993; Clapham, 1995). A number of hormones, neurotransmitters, and growth factors stimulate IP_3 formation, which, in turn, activates the IP_3 receptor Ca^{2+} release channels located predominantly in the ER. Calcium signals driven by IP_3 receptors were described first as global increases of $[Ca^{2+}]_c$, which often manifested in the form of frequency-modulated Ca^{2+} oscillations propagating throughout the cell as calcium waves (reviewed in Cobbold and Cuthbertson, 1990; Berridge, 1993; Petersen *et al.*, 1994; Clapham, 1995; Thomas *et al.*, 1996). Thus, the IP_3-dependent calcium signaling involves conversion of an analog extracellular signal, the concentration of agonist into a digital intracellular signal, the frequency of $[Ca^{2+}]_c$ transients. Furthermore, oscillations of $[Ca^{2+}]_c$ were found to be characteristic and reproducible for each cell with respect to amplitude, frequency (Prentki *et al.*, 1988), and pattern of spatial propagation (Thomas *et al.*, 1991).

The uniform $[Ca^{2+}]_c$ spikes appear to be close relatives of the neuronal action potentials and, indeed, regulation of the IP_3 receptors involves cooperativity, positive feedback, inactivation, and reactivation which are fundamental elements of the electrical excitability. Global $[Ca^{2+}]_c$ signals mediated by the IP_3 receptors have been suggested to result from spatially and temporally coordinated recruitment of subcellular release

units (Parker *et al.*, 1996; Bootman *et al.*, 1997). Because of the large amount of Ca^{2+} buffering proteins and Ca^{2+} transport organelles, the cytoplasm is a relatively poor passive conductor for Ca^{2+} increases and conduction of IP_3-induced Ca^{2+} signals is an active, self-propagating process. Recently, elementary events of IP_3 receptor-driven $[Ca^{2+}]_c$ signals have been resolved as Ca^{2+} "sparks," "puffs," and "blips." These signals are believed to represent Ca^{2+} responses associated with activation of one or a few IP_3 receptors (Yao *et al.*, 1995; Parker and Yao, 1996; Reber and Schindelholz, 1996; Bootman and Berridge, 1996; Horne and Meyer, 1997). During the brief periods of channel opening at the sites of the elementary Ca^{2+} release events, the local concentration of $[Ca^{2+}]$ rises to high levels before dissipating into the surrounding cytoplasm. Within the microdomain of the elementary event, the high levels of Ca^{2+} may result in rapid and spatially limited changes in the activity of Ca^{2+}-regulated processes, which are less sensitive to Ca^{2+} than the processes controlled by the global Ca^{2+} signals. Growing evidence supports that local spatial and temporal patterns of calcium signals may account for selective activation of specific processes (reviewed in Berridge, 1997; Putney, 1998).

PROPAGATION OF $[Ca^{2+}]$ SIGNALS INTO THE MITOCHONDRIA

The outer mitochondrial membrane is readily permeable to small molecules, whereas the inner membrane has very low permeability to Ca^{2+}. Uptake of Ca^{2+} is mediated by a mechanism (uniporter) that facilitates diffusion of Ca^{2+} down its electrochemical gradient and does not couple the transport of Ca^{2+} to that of any other ion (Fig. 2). As such, the properties of mitochondrial Ca^{2+} uptake are similar to Ca^{2+} fluxes via ion channels. Mitochondrial Ca^{2+} uptake is driven by the $\Delta\Psi_m$ that is maintained by extrusion of protons, either coupled through the electron transport system to substrate oxidation or mediated by the F_1-ATPase using energy from ATP hydrolysis. Although mitochondrial Ca^{2+} uptake is very slow at submicromolar $[Ca^{2+}]$, large amounts of Ca^{2+} can be taken up and stored by the mitochondria at higher $[Ca^{2+}]$ levels (K_d approx. 10 μM for Ca^{2+}). Recently, a new Ca^{2+} uptake mechanism, rapid uptake mode, has also been described that shows rapid activation and inactivation at $[Ca^{2+}]$ elevations in the submicromolar range (Sparagna *et al.*, 1995). The rapid Ca^{2+} uptake may correspond to a specific conformation of the uniporter. Egress of mitochondrial Ca^{2+} occurs through Na^+-dependent and -independent carriers (Fig. 2). Efflux of mitochondrial Ca^{2+} may also be mediated by the permeability transition pore (PTP) (Fig. 2). Remarkably, control of the Ca^{2+} uniporter and the PTP is complex and involves regulatory sites for Ca^{2+}. (For review articles on mitochondrial Ca^{2+} transport, see Gunter and Pfeiffer, 1990; Gunter *et al.*, 1994, 1998; Pozzan *et al.*, 1994; Jouaville *et al.*, 1998.)

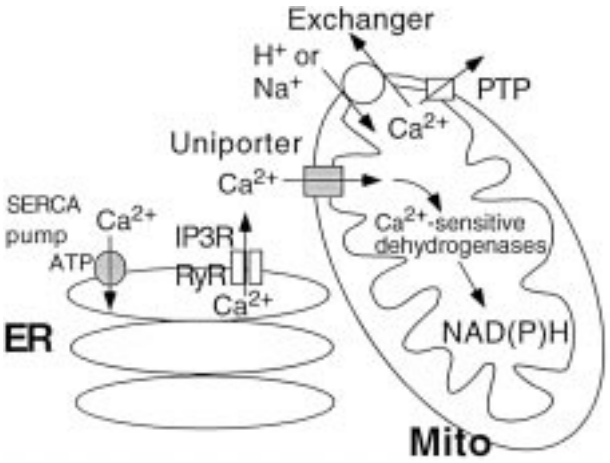

Fig. 2. Pathways of Ca^{2+} transport by ER and mitochondrial Ca^{2+} stores. Key to abbreviations: IP3R, IP_3 receptor Ca^{2+} channel; RyR, ryanodine receptor Ca^{2+} release channel; SERCA pump, sarcoendoplasmic reticulum Ca^{2+} ATPase; PTP, mitochondrial permeability transition pore. Ca^{2+} influx mechanisms are shown with filled symbols, whereas Ca^{2+} efflux pathways are shown with hollow symbols.

Since physiological elevations of global $[Ca^{2+}]_c$ evoked by various stimuli peak at submicromolar $[Ca^{2+}]$ and mitochondrial Ca^{2+} uptake is activated at higher $[Ca^{2+}]$, the role of mitochondria was believed to be limited to pathological conditions in which larger increases in $[Ca^{2+}]_c$ may occur. Using aequorin targeted to the mitochondria for direct measurement of $[Ca^{2+}]_m$, Rizzuto, Pozzan, and co-workers demonstrated that mitochondria undergo large increases of $[Ca^{2+}]_m$ following stimulation by IP_3-linked agonists in a wide variety of cell types (Rizzuto *et al.*, 1993, 1994, 1998). A similar result was obtained using fluorescent indicators compartmentalized in the mitochondria (Hajnoczky *et al.*, 1995; Donnadieu and Bourguignon, 1996; Jou *et al.*, 1996; Simpson and Russell, 1996). Prior to stimulation, $[Ca^{2+}]_m$ is close to $[Ca^{2+}]_c$ (100–300 nM), whereas the elevations of $[Ca^{2+}]_m$ are larger than $[Ca^{2+}]_c$ spikes evoked by IP_3-linked stimuli (3–20 μM versus 0.5–1 μM) (Rizzuto *et al.*, 1993; Rutter *et al.*,

1996; Csordás *et al.*, 1999). It has also been demonstrated that increases of the global $[Ca^{2+}]_c$ caused by IP_3-linked hormones or by IP_3 are associated with larger increases of $[Ca^{2+}]_m$ than comparable elevations of $[Ca^{2+}]_c$ evoked by inhibition of the ER Ca^{2+} pump in intact cells or by Ca^{2+} addition to permeabilized cells (Rizzuto *et al.*, 1993, 1994; Hajnoczky *et al.*, 1995; Csordas *et al.*, 1999). It has been postulated that the highly efficient transmission of IP_3-induced Ca^{2+} release into the mitochondria is achieved by either: (1) generation of a localized large increase of $[Ca^{2+}]_c$ near to the mitochondria; or (2) an IP_3-dependent conformational change of the uniporter; or (3) utilizing selectively the rapid Ca^{2+} uptake mode. Data obtained using subcellular systems to monitor $[Ca^{2+}]_m$ suggest that a conformational change of the uniporter evoked by IP_3 or a metabolite of IP_3 independent of the Ca^{2+} release or selective activation of the rapid Ca^{2+} uptake mode does not account for the large stimulation of mitochondrial Ca^{2+} uptake that is associated with IP_3-induced Ca^{2+} release (Rizzuto *et al.*, 1994; Csordas *et al.*, 1999). Furthermore, growing evidence argues for the idea that a local Ca^{2+} signal transmission exists between IP_3 receptors and mitochondrial Ca^{2+} uptake sites. Close apposition of ER and mitochondrial membranes has been shown by multiple approaches (Shore and Tata, 1977; Simpson *et al.*, 1997; Mannella *et al.*, 1998; Rizzuto *et al.*, 1998) and there are reports demonstrating clusters of IP_3 receptors in ER membranes facing mitochondria (Maeda *et al.*, 1989; Mignery *et al.*, 1989; Satoh *et al.*, 1990). It has also been shown that the mitochondrial redox response evoked by Ca^{2+}-mobilizing agonists occurs as a spatially organized wave that closely follows the kinetics of $[Ca^{2+}]_c$ wave propagation (Hajnoczky *et al.*, 1995). This result suggests that the $[Ca^{2+}]_m$ and redox responses are controlled locally at the subcellular sites of Ca^{2+} mobilization. Furthermore, measurements of $[Ca^{2+}]$ with aequorin targeted to the intermembrane space demonstrated $[Ca^{2+}]$ increases that exceed changes of $[Ca^{2+}]_c$ (Rizzuto *et al.*, 1998). Taken together, these data support the idea that IP_3 leads to activation of mitochondrial Ca^{2+} uptake via generation of a localized large increase of $[Ca^{2+}]_c$ near the mitochondria.

The link between IP_3 receptors and a mitochondrion may be established by two fundamentally different architectures at the level of coupling between individual IP_3 receptors and mitochondrial Ca^{2+} uptake sites. Mitochondrial Ca^{2+} uptake sites could be activated independently of each other by the Ca^{2+} release through single IP_3 receptors, analogous to the recruitment of Ca^{2+} release through ryanodine receptors by Ca^{2+} entry through single L-type Ca^{2+} channels in the heart (Lopez-Lopez *et al.*, 1995). Alternatively, populations of mitochondrial Ca^{2+} uptake sites could interact with populations of IP_3 receptors similarly to the transmission in synapses. Recent data suggest cooperation between IP_3 receptors in activation of mitochondrial Ca^{2+} uptake, that would not be expected if IP_3 receptors and mitochondrial Ca^{2+} uptake sites are coupled on a one-to-one basis (Csordas *et al.*, 1999). In addition, imaging of the close contacts between ER and mitochondria (Rizzuto *et al.*, 1998) and calculation of the local $[Ca^{2+}]$ sensed by the mitochondrial Ca^{2+} uptake sites (Csordas *et al.*, 1999) yielded data to suggest that the average distance between IP_3 receptors and mitochondrial Ca^{2+} uptake sites is not in the $<$ 20-nm range, but may be in the 100-nm range. Microdomains of this size can result from the superposition of the Ca^{2+} contributions of several nearby channels (Dunlap *et al.*, 1995; Borst and Sakmann, 1996; Cooper *et al.*, 1996). Taken together, these data suggest that Ca^{2+} release through multiple IP_3 receptors is integrated at the level of individual mitochondrial Ca^{2+} uptake sites.

Notably, a recent study from our laboratory described several functional properties of the Ca^{2+} signal transmission between ER and mitochondria that are also fundamental features of the quantal synaptic transmission (del Castillo and Katz, 1954; Katz, 1969), including transient microdomains of high transmitter concentration, saturation of targets by the transmitter, connection of multiple release sites to each target, and quantal transmission. As such, the functional architecture of connections between subcellular organelles mimics the organization of intercellular synaptic junctions. The corresponding elements of subcellular Ca^{2+} signal transmission and synaptic transmission and their common functional features are listed in Table I.

Several studies have been concerned with the question whether all or only subsets of mitochondria are supported by local Ca^{2+} signal transmission. Spatially resolved $[Ca^{2+}]$ and NAD(P)H measurements in single hepatocytes demonstrated that propagation of the $[Ca^{2+}]_c$ wave into the mitochondria shows constant efficiency along the entire path of wave propagation (Hajnoczky *et al.*, 1995). Using mitochondrially targeted aequorin, 30% of the total cellular mitochondrial pool was calculated to be highly responsive to IP_3-linked stimuli in populations of MH75 cells (Rizzuto *et al.*, 1994), whereas in individual CHO cells an essentially homogenous increase of $[Ca^{2+}]_m$ was observed over the cells (Rutter *et al.*, 1996). Using compartmentalized fluorescent Ca^{2+} indicators, all mitochondrial

Table I. Functional Similarities between Calcium Signal Transmission from ER to Mitochondria and Synaptic Transmission[a]

	Calcium signal transmission between ER[a] and mitochondria	Synaptic transmission between cells
Activator	IP_3[b]	Action potential
Messenger	Ca^{2+}	Neurotransmitter
Source	ER through IP3R[c]	Synaptic vesicle
Target	Ca^{2+}-uniporter	Neurotransmitter receptor
Common features of functional organization	Microdomains of high messenger concentration which rapidly dissipate	
	Maximal activation of the targets	
	Quantal pattern of transmission	
	Multiple source units communicate with each target	
	Constitutive/nonvesicular release of the messenger is poorly detected	

[a] ER, endoplasmic reticulum.
[b] IP_3, inositol-1,4,5-trisphosphate.
[c] IP3R, IP_3 receptor Ca^{2+} channel.
[d] Reproduced with modification from Csordas, G., Thomas, A.P., and Hajnoczky, G. (1999), by permission of Oxford University Press.

Ca^{2+} uptake sites were shown to become fully activated during Ca^{2+} release induced by maximally effective IP_3 in permeabilized mast cells (Csordas *et al.*, 1999). Recent studies using aequorin targeted to the intermembrane space indicated that only a small fraction of the mitochondrial inner membrane is exposed to high $[Ca^{2+}]$ microdomains in HeLa cells (Rizzuto *et al.*, 1998). Clearly, different distribution and density of IP_3 receptors in various cells or differences in the spatiotemporal pattern of IP_3 receptor activation during stimulation with IP_3-linked stimuli may account for cell-specific mitochondrial responses.

Taken together, the data described above have verified the concept that IP_3 receptor mediated increases of $[Ca^{2+}]_m$ utilize local signal transmission, but it will be challenging to determine the precise control of Ca^{2+} transport between ER release sites and mitochondrial Ca^{2+} uptake sites. Nevertheless, the results that mitochondrial Ca^{2+} uptake is closely coupled to the rising phase of the IP_3 receptor-mediated $[Ca^{2+}]_c$ response and that optimal activation of mitochondrial Ca^{2+} uptake is achieved by recruitment of multiple elementary events, explain why calcium oscillations evoked by synchronized periodic activation of IP_3 receptors are particularly effective in establishing dynamic control over mitochondrial function.

CONTROL OF CELLULAR FUNCTIONS BY MITOCHONDRIAL $[Ca^{2+}]$ SIGNALS

This section is concerned with three major issues of intracellular calcium signaling, which involve generation of mitochondrial calcium signals: (i) control of energy metabolism, (ii) control of cell death and (iii) cytosolic Ca^{2+} homeostasis.

Control of Mitochondrial Energy Metabolism by $[Ca^{2+}]$ Oscillations

Three mitochondrial dehydrogenases are known to be regulated by $[Ca^{2+}]_m$ in the hundred nanomolar to micromolar range measured in the matrix in healthy intact cells (Denton and McCormack, 1980; Hansford, 1980; McCormack *et al.*, 1990). Isocitrate dehydrogenase (ICDH) and oxoglutarate dehydrogenase (OGDH) are allosterically regulated by Ca^{2+}. Pyruvate dehydrogenase (PDH) is inactivated by phosphorylation and activation by Ca^{2+} is mediated by a Ca^{2+}-stimulated phosphatase. All three CSMDHs catalyze the reduction of NAD to reduced nicotinamide adenine dinucleotide (NADH), and the OGDH and PDH enzyme complexes contain a flavoprotein, lipoamide dehydrogenase, that transfers the reducing equivalents via reduced flavin adenine dinucleotide ($FADH_2$) to NADH. NADH is a key intermediate providing reducing equivalents to the mitochondrial respiratory chain for ATP synthesis.

Single-cell imaging studies that took advantage of the fact that the activity of CSMDH can be monitored fluorometrically through changes in the redox state of their flavin and pyridine nucleotide cofactors revealed that the pulsatile release of Ca^{2+} underlying $[Ca^{2+}]_c$ oscillations is giving rise to associated oscillations of CSMDH (Pralong *et al.*, 1994; Hajnoczky *et al.*, 1995; Rohacs *et al.*, 1997; Robb-Gaspers *et al.*, 1998a,b). In hepatocytes, $[Ca^{2+}]_c$ oscillations with frequencies in the upper range evoked sustained elevations of

NAD(P)H and $FADH_2$, whereas sustained $[Ca^{2+}]_c$ increases were associated with only a transient redox response (Hajnoczky *et al.*, 1995). These results suggested that the frequency-modulated $[Ca^{2+}]$ signals are translated into a time-averaged redox response, the mean amplitude of which increases with agonist dose. Decay of the redox response during sustained $[Ca^{2+}]_c$ responses evoked by maximal stimulation is explained by the fact that $[Ca^{2+}]_m$ shows only a transient elevation and so there is no sustained $[Ca^{2+}]_m$ signal to maintain the activation of CSMDH (Hajnoczky *et al.*, 1995). The divergence of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ signals at maximal stimulation may be explained by the decay of the locally high $[Ca^{2+}]_c$ once the initial Ca^{2+} mobilization phase is complete. Taken together, control of mitochondrial metabolism through $[Ca^{2+}]_m$ is tuned to the oscillatory range of $[Ca^{2+}]_c$ signals and this is due to efficient propagation of the initial bursts of IP_3-induced Ca^{2+} release to the mitochondria, whereas the sustained phase of IP_3-dependent Ca^{2+} mobilization and some other forms of $[Ca^{2+}]_c$ elevations are actually tuned out. Notably, CSMDH are also sensitive to regulation by metabolites that may contribute to the complexity of the mitochondrial metabolic regulation described in intact cells exposed to IP_3-linked agonists (Robb-Gaspers *et al.*, 1998b). Furthermore, depolarization elicited by the electrogenic mitochondrial Ca^{2+} uptake (Duchen *et al.*, 1993; Loew *et al.*, 1994; Rutter *et al.*, 1996; Robb-Gaspers *et al.*, 1998b) may also affect ATP formation.

Control of Cell Death

Typically, mitochondrial metabolism is the major source of cellular ATP and if mitochondrial ATP production is insufficient, necrotic cell death occurs (for review see Lemasters *et al.*, 1998). Recently, mitochondria have also been established to play a specific and fundamental role in apoptotic cell death by releasing activators of the caspase cascade (for recent review see Bernardi, 1996; Kroemer *et al.*, 1997; 1998; Reed, 1997; Green and Kroemer, 1998; Green and Reed, 1998; Ichas and Mazat, 1998; Skulachev 1998). Remarkably, opening of the PTP appears to be involved in the mitochondrial phase of both necrotic and apoptotic cell death. Since elevation of $[Ca^{2+}]_m$ is a main activator of the PTP, the role of mitochondrial Ca^{2+} in execution of cell death has been receiving considerable attention.

Opening of PTP yields a rapid increase in the mitochondrial inner membrane permeability to molecules with mass up to 1500 Da, which, in turn, results in dissipation of $\Delta\Psi_m$, uncoupling of oxidative phosphorylation, release of ions and metabolic intermediates, and mitochondrial swelling (for review see Zoratti and Szabo, 1995). Growing evidence suggests that opening of PTP is important in release of apoptotic factors, *e.g.*, cytochrome *c* and apoptosis-inducing factor, which are located in the intermembrane space (for review see Kroemer *et al.*, 1997; 1998; Reed, 1997; Green and Kroemer, 1998; Green and Reed, 1998), although cytochrome *c* release independent of PTP has also been reported (*e.g.*, Li *et al.*, 1998). Recently, a low-conductance/transient activation mode of PTP opening has also been proposed that yields rapid equilibration of molecules with mass up to 300 Da. Release of the proapoptotic factors and swelling are not associated with activation of the low-conductance state of PTP (Ichas *et al.*, 1997; Ichas and Mazat, 1998; Ichas *et al*. 1999). Activation of PTP is promoted by elevations of $[Ca^{2+}]_m$, high pH (particularly important in activation of the low-conductance mode), depolarization, and oxidant agents, whereas protective effect is exerted by ADP, Mg^{2+}, $[Ca^{2+}]_c$ (for review see Zoratti and Szabo, 1995; Bernardi and Petronilli, 1996).

Since activation of PTP appears to play a fundamental role in priming of both necrotic and apoptotic cell death, it is possible that differential regulation of PTP is required to drive the two different death pathways. A major factor in discrimination between necrosis and apoptosis may be cellular ATP. If ATP is low, the apoptotic program does not proceed. Importantly, opening of PTP yields impaired mitochondrial ATP production (loss of H^+ gradient and cytochrome *c*) and, furthermore, opening of PTP leads to mitochondrial ATP consumption, because of the reverse operation of the F_1F_0-ATPase. To carry out the apoptotic program that is initiated by release of cytochrome *c* or apoptosis-inducing factor during PTP opening, ATP should be provided from an alternative source (glycolysis) or the metabolic function of mitochondria should return. It has also been proposed that if PTP opening involves only a subset of mitochondria, these may provide a sufficient apoptotic signal to ignite the apoptotic machinery, whereas the remaining normal mitochondria may maintain ATP (Lemasters *et al.*, 1998).

Large amounts of Ca^{2+} loaded into mitochondria have been shown to evoke PTP opening (for review see Gunter *et al.*, 1994; Zoratti and Szabo, 1995; Bernardi, 1996; Bernardi and Petronilli, 1996) and cytochrome

c release (Andreyev *et al.*, 1998; Petit *et al.*, 1998; Yang and Cortopassi, 1998). Although mitochondrial swelling in hepatocytes stimulated with Ca^{2+}-mobilizing agonists has also been observed (Halestrap *et al.*, 1990), it has been demonstrated in many cell types that in health, IP_3 receptor-mediated Ca^{2+} responses are reversed by Ca^{2+} efflux through the exchangers (Rizzuto *et al.*, 1994) or via PTP in a low-conductance state (Ichas *et al.*, 1997). These data led to the concept that rapid and brief mitochondrial $[Ca^{2+}]$ signals evoked by IP_3-linked agonists are utilized in the control of cellular energy metabolism or are important in organization of the cytosolic calcium signal (Jouaville *et al.*, 1995; Budd and Nicholls, 1996; Simpson and Russell, 1996; Ichas *et al.*, 1997; Babcock and Hille, 1998; Ichas and Mazat, 1998), but do not evoke PTP opening and cytochrome *c* release. By contrast, prolonged and substantial Ca^{2+} loading of the mitochondria evoked by sustained Ca^{2+} entry or depletion of ER Ca^{2+} stores may yield cell death with (Hoek *et al.*, 1997) or without (Nicholls and Budd, 1998a,b) PTP opening and release of apoptotic factors. In this model, proceeding of cells on the apoptotic track can be envisioned if extramitochondrial sources maintain ATP or PTP opening occurs only in a subset of the mitochondria. Considering that PTP is controlled by a number of factors (Ca^{2+}, pH, free radicals, adenine nucleotides) it may also function as a coincidence detector. Data from our laboratory suggest that stimulation of HepG2 cells with IP_3-linked agonists triggers the mitochondrial phase of apoptosis in cells exposed to proapoptotic stimuli (C2 ceramide or staurosporin) but not in naive cells (Szalai, Krishnamurthy and Hajnoczky, unpublished data). It is speculated that utilization of IP_3 receptor-driven $[Ca^{2+}]$ spikes in activation of the release of apoptotic factors may increase the fidelity of the apoptotic pathway provided that the decay of the $[Ca^{2+}]$ transients is followed by resealing of PTP and by recovery of mitochondrial ATP production.

An additional mechanism to control participation of intracellular Ca^{2+} stores in execution of cell death involves interactions with proteins of the Bcl-2 family (Baffy *et al.*, 1993; Murphy *et al.*, 1996; Reed, 1997). PTP-mediated cytochrome *c* release may play an important role in the effect exerted by proapoptotic (*e.g.*, BAX, Oltvai *et al.*, 1993; Pastorino *et al.*, 1998; Marzo *et al.*, 1998; but BID, Li *et al.*, 1998) and antiapoptotic members of the Bcl-2 family (Hockenbery *et al.*, 1990) on the mitochondria. Once again, the signal deriving from interactions with Bcl-2 proteins may be integrated with IP_3 receptor-mediated $[Ca^{2+}]_m$ signals at the level of PTP and coincidence of subtreshold signals may determine the fate of the cell.

Cytosolic Calcium Regulation

Demonstration of large increases in $[Ca^{2+}]_m$ evoked by IP_3-linked agonists and the emerging concept of local Ca^{2+} signaling between ER Ca^{2+} release sites and mitochondrial Ca^{2+} uptake sites have stimulated research to determine whether mitochondrial Ca^{2+} uptake plays a role in shaping IP_3 receptor-mediated $[Ca^{2+}]_c$ signals. Interestingly, inhibitors of mitochondrial Ca^{2+} uptake have been shown to abolish IP_3-mediated $[Ca^{2+}]_c$ oscillations in gonadotropes (Hehl *et al.*, 1996), whereas less dramatic effects appear in some other systems (see below). The simplest mechanism by which the mitochondrial Ca^{2+} transport pathways can modify $[Ca^{2+}]_c$ signals is by acting as a slow buffer, which accumulates Ca^{2+} during rapid $[Ca^{2+}]_c$ increases and then returns the Ca^{2+} as $[Ca^{2+}]_c$ declines. In this way the mitochondria can blunt and prolong a $[Ca^{2+}]_c$ transient, as occurs during depolarization-induced Ca^{2+} influx in chromaffin cells (Babcock *et al.*, 1997). A more complex interaction between Ca^{2+} release sites and mitochondria was suggested by the observation that mitochondrial energization in *Xenopus* oocytes enhances the organization of IP_3-activated $[Ca^{2+}]_c$ waves by decreasing frequency and increasing the amplitude of Ca^{2+} release (Jouaville *et al.*, 1995). Studies of IP_3-induced ER $[Ca^{2+}]$ ($[Ca^{2+}]_{ER}$) responses provided evidence that mitochondria can directly regulate the Ca^{2+} release function of the IP_3 receptors by modulating the feedback effects of cytosolic Ca^{2+} (Landolfi *et al.*, 1998; Hajnoczky *et al.*, 1999). Specifically, we observed that mitochondrial Ca^{2+} uptake suppresses the positive feedback effects of $[Ca^{2+}]_c$ during IP_3-induced Ca^{2+} release in permeabilized hepatocytes. By decreasing the IP_3 receptor excitability, mitochondrial Ca^{2+} uptake may yield a lower Ca^{2+} wave frequency and ensure that a greater proportion of IP_3 receptors are in the resting state available to contribute to Ca^{2+} release when the activation threshold is finally achieved at the Ca^{2+} wave front. In oligodendrocytes, mitochondria appear to be selectively localized at sites of Ca^{2+} wave amplification (Simpson and Russell, 1996; Simpson *et al.*, 1997). This observation could reflect a role for mitochondrial Ca^{2+}-induced Ca^{2+} release, whereby the accumulation of $[Ca^{2+}]_m$ elicits mitochondrial depolarization and consequent Ca^{2+} release (Ichas *et al.*, 1997).

Alternatively, the spatial and temporal properties of mitochondrial Ca^{2+} uptake in the oligodendrocyte may act predominantly to suppress the negative feedback effects of $[Ca^{2+}]_c$. Importantly, mitochondria also decrease Ca^{2+}-dependent inactivation of the plasma membrane Ca^{2+} current initiated by depletion of the ER Ca^{2+} store (Hoth *et al.*, 1997). Taken together, it appears that mitochondria can have a number of important effects on cytosolic Ca^{2+} signaling. These effects are not limited to simple Ca^{2+} buffering, but include direct modulation of the feedback effects of $[Ca^{2+}]_c$ on its own release. Since Ca^{2+} feedback is complex, positive as well as negative feedback effects occur in dose- and time-dependent manner, the spatial distribution and temporal features of mitochondrial Ca^{2+} uptake may determine the mitochondrial control over organization of calcium signals.

CONCLUSIONS

The main goal of this review was to summarize recent research on the mechanisms underlying translation of IP_3-dependent cytosolic calcium signals into mitochondrial responses. The first step of the decoding process is propagation of the $[Ca^{2+}]_c$ rise into the mitochondrial matrix. Mitochondrial Ca^{2+} uptake is supported by a local control of $[Ca^{2+}]$ between IP_3 receptors and mitochondrial Ca^{2+} uptake sites that are located close to each other. Although the precise mechanism of the Ca^{2+} transfer between individual release and uptake sites has not been resolved, recent evidence suggests that Ca^{2+} signal transmission between intracellular organelles can utilize a closely related functional architecture to that used for synaptic signal propagation between cells. Calcium ion entering the mitochondria may control mitochondrial metabolism, but it may have a role in initiation of the process leading to cell death and it may also provide feedback mechanisms to modulate $[Ca^{2+}]_c$ signals. The mitochondrial targets of Ca^{2+} appear to display distinct Ca^{2+} sensitivity and are also affected by other signals (*e.g.*, metabolic factors, other ions, $\Delta\Psi_m$, free radicals). Thus, differential regulation of mitochondrial functions by $[Ca^{2+}]_m$ signals may utilize the quantitative properties of Ca^{2+} uptake and coincidence detection as well.

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